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Chrysin attenuates liver fibrosis and hepatic stellate cell activation through TGF-β/Smad signaling pathway

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ABSTRACT

We investigated the protective effect of chrysin on chronic liver fibrosis in mice and the potential mechanism underlying TGF-β1-mediated hepatic stellate cells (HSCs) activation on fibrogenesis.

Experimental fibrosis was established by intraperitoneal injection of mice with 20% v/v, 2 ml/kg CCl_4 twice a week, for 7 weeks. Mice were orally treated with 3 doses of chrysin (50, 100 and 200 mg/kg) or with vehicle as control. For the assessment of the spontaneous reversion of fibrosis, CCl_4 treated animals were investigated after two weeks of recovery time. Silymarin was used as standard hepatoprotective flavonoid.

Histopathological investigations showed that hepatic fibrosis grade was markedly reduced in the chrysin groups compared to the fibrotic one. Moreover, CCl_4 activated HSCs induced an upregulation of smooth muscle actin (α-SMA), an increased number of TGF-β1 immunopositive cells and marked up-regulation of TGF-β1. α-SMA and TGF-β1 levels were significantly reduced in all chrysin treated groups in a dose-dependent manner, whereas the level of spontaneous reversal of fibrosis was lower compared to all flavonoid treated groups. Liver mRNA levels of Smad 2 in the 50, 100 and 200 mg/kg chrysin treated groups were significantly reduced by about 88.54%, 92.15% and 95.56% of the corresponding levels in the fibrosis mice group. The results were similar for mRNA levels of Smad 3. The protective response to silymarin was almost similar to that seen with the highest doses of chrysin.

In this study, we have shown that chrysin has the efficacy to reverse CCl_4-stimulated liver fibrosis by inhibition of HSCs activation and proliferation through TGF-β1/Smad pathway. These results suggest that chrysin may be useful in stopping or reversing the progression of liver fibrosis and might offer the possibility to develop a new therapeutic drug, useful in treatment of chronic liver diseases.

1. Introduction

Liver fibrosis is a response to chronic liver injury caused by a variety of etiological factors including drug abuse, alcohol consumption, hepatitis B and C or toxic injuries [1]. Upon liver injury, hepatic stellate cells (HSCs) are activated, followed by cytokine and chemokine release, which induce trans-differentiation into myofibroblast-like cells. This step is characterized by the appearance of smooth muscle α-actin (α-SMA) and procollagen-I, finally resulting in increased production and accumulation of extracellular matrix (ECM) components and collagen. In the pathogenesis of liver fibrosis, transforming growth factor-β1 (TGF-β1) has been recognized as a key activator of HSCs [2]. It acts by activating Smad signaling pathway and is produced by Kupffer cells, endothelial cells and hepatocytes [3]. Moreover, it also up-regulates TGF-β1 determining the synthesis of proteins associated with ECM [4].

The process of liver fibrosis can be reversible, whereas cirrhosis, the end-stage consequence of fibrosis, is generally irreversible [5]. Therefore, it is important to provide potent anti-fibrotic agents for the effective treatment of hepatic injury and fibrosis.

Several studies have been conducted to find natural products, able to prevent, retard or reverse liver fibrosis progression [6].

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Berberine exerts hepatoprotective effects possibly via activation of AMP-activated protein kinase (AMPK), blocking Nox4 and Akt expression [7], whereas resveratrol acts by restraining oxidative damage [8]. Curcumin alleviates intrahepatic angiogenesis and capilarization of the sinusoid, through suppressing multiple proangiogenic factors and reducing liver fibrosis [9]. Other studies showed that garlic extracts [10], bilberry fruit extracts [11], mistletoe alkaloids [12] attenuated liver fibrosis by inhibiting the TGF-β1/Smad pathway.

Chrysin (5,7-dihydroxyflavone) is the major active component of honey, propolis and many plant extracts. The medicinal value of chrysin has been recognized. It shows anti-inflammatory [13], anti-cancer [14], anti-allergic [15] and antioxidant [16,17] activities. However, the mechanistic action of chrysin in liver fibrosis remains to be determined.

In view of the ability of chrysin to alleviate or reverse liver fibrosis, we investigated its effects on TGF-β1-mediated HSCs activation caused by carbon tetrachloride (CCl_4) in mice.

2. Material and methods

2.1. Materials

Chrysin 97%, silymarin 98% and carboxymethyl cellulose were purchased from Sigma–Aldrich (Germany). Anti-TGF-β1, α-SMA and Smad 2/3 antibodies were supplied from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Novocastra kit for immunohistochemistry was purchased from Leica Microsystems (Germany).

2.2. Animals

Male CD1 mice from our breeding colony, 4 months old, were divided into 7 groups with 10 animals per group. The mice were fed a standard rodent diet and were maintained at 12 h light/dark cycle at constant temperature and humidity. All experimental procedures were approved by the Ethical Committee of "Vasile Goldis" Western University of Arad.

2.3. CCl_4-induced liver fibrosis

The chrysin was administered by gavage in graded doses ranging up to 4 g/kg body weight and the mice were observed for signs of toxicity and mortality for 30 days afterwards. The flavonoid was found to be practically non-toxic when administered orally to mice and its LD50 value was found to be higher than 4 g/kg body weight [data not shown].

For our experiment, the doses were chosen on the basis of previous studies concerning antioxidant or hepatoprotective protection of chrysin [14,18].

Male CD1 mice were treated intraperitoneally (i.p.) with CCl_4 dissolved in olive oil (20% v/v, 2 ml/kg), twice a week for 7 weeks and sacrificed seventy-two hours after the last injection for confirmation of liver fibrosis (group 2). Spontaneous resolution of hepatic fibrosis was investigated in CCl_4 treated animals after two weeks of recovery (group 3). In order to evaluate the antifibrotic effect of chrysin, the treatment was started at the end of the 7 weeks CCl_4 treatment using doses of 50, 100, or 200 mg/kg daily for 14 days (groups 4, 5, and 6). For oral administration, chrysin powder was dissolved in 0.7% carboxymethyl cellulose (CMC). Mice belonging to groups 1 (control), 2 and 3 received 0.7% CMC solution instead of chrysin. Silymarin in 0.7% CMC at 200 mg/kg dose was used as positive control (group 7). Animals were sacrificed 24 h after the last dose of chrysin, silymarin or CMC by cervical dislocation (1, 3, 4, 5, 6, 7 groups). Tissue samples were used for biochemical and molecular analysis, or were preserved in a 4% phosphate buffered formaldehyde solution to obtain histological sections.

2.4. Histopathology

The fixed tissues were embedded in paraffin, cut into 5 μm thick sections and stained with hematoxylin&eosin (H&E) or Fouchet van Gieson according to the protocol provided with the Bio-Optica staining kit. Sections were examined using an Olympus BX43 microscope and photographed using a digital camera (Olympus XC30).

Morphometric analysis for fibrosis quantification was performed using five random low-power images per animal at 50× magnification. The percentages of areas with collagen deposition were quantified using NIH ImageJ software.

2.5. Immunohistochemistry

Liver sections were deparaffinized in toluene and rehydrated prior to epitope retrieval in Novocastra solution (Leica Biosystems, Germany). After neutralization of the endogenous peroxidase with 3% H_2O_2 for 10 min the sections were incubated with blocking solution overnight at 4 °C with anti-TGF-β1, Smad 2/3 or α-SMA antibodies (1:100, dilution). Detection was performed using a polymer detection system (Novolink max Polymer detection system, Novocastra Leica Biosystems) and DAB (3,3′-Diaminobenzidine, Novocastra Leica Biosystems) as chromogenic substrate. Hematoxylin staining was applied before dehydration and mounting. Images were acquired by light microscopy (Olympus BX43, Hamburg, Germany).

2.6. Real-time RT-PCR analysis

Liver tissue samples were stored in RNA later solution (ThermoScientific) at −80 °C. The tissue was homogenized and RNA was isolated using SV Total RNA Isolation System (Promega), according to the manufacturer's instructions. The quantity and quality of purified RNA was assessed using a NanoDrop 8000 spectrophotometer (Thermo Scientific). 2 μg total RNA was transcribed into cDNA using First Strand cDNA Synthesis Kit (Thermo Scientific). Conditions for the reverse transcriptase reaction were: 25 °C for 5 min, 37 °C for 60 min and 70 °C for 5 min. Real-time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Life Technologies) with Mx3000P™ real-time PCR system. All samples were run in triplicate. The primers sequences and all experimental validation data were accessed from the Primer Bank website, http://pga.mgh.harvard.edu/primerbank/. The amount of mRNA investigated was normalized to GAPDH. Relative expression changes were determined using the 2 ΔΔ C(T) method [19].

Primers were synthesized by Eurogentec, Belgium (Table 1).

2.7. Statistical analysis

Statistical analysis was conducted with a one-way ANOVA using Stata 13 software (StataCorp LP, Texas, USA). A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Chrysin treatment ameliorates hepatic lesions in the livers of mice with CCl_4-induced fibrosis

Histopathologic evaluation is regarded as the proper method for assessing the presence and severity of hepatic fibrosis. In order to determine the extent of hepatic fibrosis, we evaluated liver sections